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Therapeutic vaccines for amyotrophic lateral sclerosis directed against disease specific epitopes of superoxide dismutase 1

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ABSTRACT

Emerging evidence suggests seeding and prion-like propagation of mutant Superoxide Dismutase 1 (SOD1) misfolding to be a potential mechanism for ALS pathogenesis and progression. Immunotargeting of misfolded SOD1 has shown positive clinical outcomes in mutant SOD1 transgenic mice. However, a major challenge in developing active immunotherapies for proteinopathies such as ALS is the design of immunogens enabling exclusive recognition of pathogenic species of a self-protein. Ideally, one would achieve a robust antibody response against the disease-misfolded protein while sparing the natively folded conformer to avoid inducing deleterious autoimmune complications, or inhibiting its normal function. Using a motor neuron disease mouse model expressing human SOD1-G37R, we herein report the immunogenicity and therapeutic efficacy of two ALS vaccines, tgG-DSE2lim and tgG-DSE5b, based on the notion that native SOD1 would undergo early unfolding in disease to present "disease specific epitopes" (DSE). Both vaccines elicited rapid, robust, and well-sustained epitope-specific antibody responses with a desirable Th2-biased immune response. Both vaccines significantly extended the life expectancy of $hSOD1^{G37R}$ mice, with tgG-DSE2lim displaying greater protection than tgG-DSE5b at earlier pre-symptomatic stage. tgG-DSE5b, but not tgG-DSE2lim, significantly delayed disease onset and appreciably slowed disease progression. This implies that conformationally distinct species of misfolded SOD1 may derive from the same mutation, thereby modifying disease phenotypes in a different fashion. Our results validate the rationale for conformation-based immuno-targeting of misfolded SOD1 as a promising therapeutic strategy to slow or even halt disease progression in familial ALS associated with SOD1 mutations, as well as a prophylactic intervention for carriers of SOD1 mutations. Our study not only provides important proof-of-principle data for the development of a safe and effective human therapeutic/prophylactic ALS vaccine against misfolded SOD1, but also predicts a great potential to extend our DSE-based vaccination approach to other types of ALS, such as those associated with TDP-43 proteinopathies.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease characterized by degeneration of motor neurons of the brain and spinal cord, leading to progressive paralysis of the affected individuals [1]. The average incidence of ALS is ~2 per 100,000 population each year, although the prevalence varies among geographic regions globally. The majority of patients die within 2–5 years of diagnosis due to progressive paralysis and eventual respiratory failure. To date, there is no cure for ALS. Up to 2017, riluzole had been the only FDA approved drug for treatment of ALS since 1995, but it extends survival by only 2–3 months [2]. Radicava, a new FDA approved drug, has been reported to slow the rate of decline in physical function by 33% (ClinicalTrials.gov Identifier, NCT01492686).

Approximately 5–10% of ALS cases are familial (fALS), ~20% of which are associated with mutations in the gene encoding copper/zinc superoxide dismutase (SOD1), a cytosolic free radical scavenger enzyme. Natively folded SOD1 exists as a homodimer via an intramolecular disulfide bond, with each monomer binding a copper/zinc ion [3–5]. Since first discovered as a pathological protein of ALS in 1993 [6], over 150 mutations in SOD1 have been identified but the precise pathomechanisms associated with SOD1

Abbreviations: DSE, disease-specific epitope; SOD1, superoxide dismutase 1; ALS, amyotrophic lateral sclerosis; tgG, truncated rabies glycoprotein G.

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mutations remain poorly understood. Some studies have suggested that mutant SOD1 imparts a neurotoxic gain-of-function linked to the propensity of mutant proteins to misfold and aggregate [7]. Therapeutic strategies have been directed to neutralizing existing misfolded SOD1 molecules [8–11].

Preclinical trials have suggested immunotherapy to be a promising therapeutic avenue for neurodegenerative diseases associated with proteinopathies, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's Disease (HD), prion diseases, and ALS [12–15]. For ALS, both passive and active immunization studies have been carried out using motor neuron disease mouse models expressing human SOD1 mutations, often with positive therapeutic outcomes [8–11]. For immunotherapeutics, active immunotherapy (vaccination) has many advantages over passive immunotherapy by providing a more sustained protection without the requirement for repeated infusion of antibodies. Moreover, a successful vaccination strategy against ALS will not only ameliorate disease course, but also provide a more economical therapy for patients requiring a lifetime of disease management, and improve their quality of life. A prophylactic ALS vaccine would also offer an early precision prevention to genetic carriers of mutant SOD1.

Active immunization for proteinopathies is complicated by tolerance of the immune system to antigens associated with self-proteins. There is also the potential danger that antibodies immunoreactive to the native isoform of the protein could have deleterious autoimmune consequences, or inhibit the function of the natively folded protein. One way to address these concerns is to target regions of the protein whose exposure for antibody binding is dependent upon the protein misfolding to the pathological conformation. These disease-specific epitopes (DSEs), presented in the context of an appropriate protein carrier system, have potential to induce antibody responses that are specific to the misfolded conformation, as we reported previously [16,17].

For the current investigation, we employed a similar strategy to direct an immune response specifically against regions of SOD1 that are only exposed upon unfolding/misfolding. These regions were selected based on data from X-ray crystal structures and molecular dynamics simulation studies [18,19], as well as "collective coordinates", a computational algorithm modeling of unfolding energy initiated from an atomic resolution protein structure as deposited in the protein data base [20]. Two DSE-based vaccines against misfolded SOD1, tgG-DSE2lim and tgG-DSE5b, were created and used to immunize a late onset ALS mouse model expressing human mutant SOD1-G37R (hSOD1^{G37R}). We report here that both vaccines display significant therapeutic efficacy alleviating disease phenotypes of hSOD1^{G37R} animals. Our study validates the rationale for our conformation specific epitope based immuno-targeting strategy as a promising treatment for fALS-SOD1 patients and potentially a prophylactic approach for carriers of SOD1 mutations.

2. Results

2.1. Designs of DSE2lim and DSE5b based vaccines

A variety of approaches has been employed to delineate the molecular basis determining the conformations of native and misfolded SOD1 mutants [4,20]. One region identified in X-ray crystal structure data from human native and ALS-associated mutant SOD1 proteins is the electrostatic loop (ESL), ^{126}LGKGGNEESTKTGNAGS^{142}, which is normally compact in native dimeric SOD1 but adopts an extended and unstructured conformation upon zinc depletion and unfolding of SOD1, as occurs in metal-deficient pathogenic SOD1 mutants [18]. This unstructured fragment inserts itself into a groove formed between the aberrantly exposed β-sheets 5 and 6 of an adjacent SOD1 molecule to form a non-native protein interaction, which can then act as a template to recruit neighbouring SOD1 molecules and ultimately assembly into larger aggregates of amyloid-like filaments [18]. Molecular dynamics simulation identified regions in SOD1 that were highly amyloidogenic for SOD1 aggregation under destabilizing conditions. One of these sequences, ^{35}IKGLTEGLHGF^{45}, forms a crossover loop between β-sheets 3 and 4 in the native SOD1 [19].

Utilizing "collective coordinates", we identified more defined fragments in the above SOD1 regions that were most likely to unfold/misfold under disease conditions, ^{132}EESTKT^{137} and ^{35}IKGLTEG^{41} [20]. Notably, we found that a linear peptide mapping to the short α-helix ^{32}EESTKT^{13} was the most potent DSE in the ESL, presumably due to unwinding of the α-helix in SOD1 misfolding. Further optimization for enhanced immunogenicity and vaccine utility was performed using a novel *in silico* epitope immunogenicity characterization program, EpIC [21]. Together, the above studies led to the core epitope sequences of our DSE2lim and DSE5b, ^{131}NEESTKTGN^{139} and ^{34}SIKGLTEG^{41}, respectively.

To induce an antibody response against a self-protein, a carrier protein with T cell epitopes is required in the composition of an effective vaccine. Our previous research in the development of peptide-based vaccines against prion proteins demonstrates that the choice of carrier protein can significantly alter the magnitude and duration of epitope-specific antibody responses, and the bias of Th1 *vs.* Th2 immune responses [22]. Specifically, we established that a truncated rabies glycoprotein, tgG, induced a strong desirable Th2-biased humoral response, as compared to other commonly used carrier proteins such as Leukotoxin (Lkt), which induced a Th1/Th2 balanced response in vaccinated mice [22]. Earlier vaccination studies using recombinant G93A-SOD1 as the immunogen showed that the magnitude of epitope-specific antibody titres was highly correlated with the clinical outcomes of vaccinated mutant SOD1 transgenic mice [9,11]. Therefore, to formulate a Th2-biased vaccine with robust and durable antibody responses, we chose tgG as the carrier in this study.

2.2. Immunogenicity of tgG-DSE2lim and tgG-DSE5b in normal C57BL/6 mice

We first set out to test the immunogenicity of tgG-DSE2lim and tgG-DSE5b vaccines in cohorts of 8 normal C57BL/6 mice. The inverted sequence of DSE2lim, NGTKTSEEN, contained a predicted N-linked glycosylation site "NGT", as confirmed by deglycosylation assay using N-glycosidase "PNGase F" (Fig. S1). To rule out potential interference of glycosylation with the immune response profile of tgG-DSE2lim, we also included a cohort vaccinated with PNGase F treated tgG-DSE2lim.

Each animal received a total of two injections at week 0 and 3. Serum samples were collected at Day 0 immediately prior to vaccination and at weeks 3, 4, 7, 10, 14, 18 and 28 (endpoint) following the initial immunization. Epitope-specific antibody responses were monitored by enzyme-linked immunosorbent assay (ELISA), and the median titre for each cohort was plotted over all time points. As shown in Fig. 1A, all three vaccines induced robust antibody responses at comparable magnitude by week 3, rising thereafter and peaking at different times. No significant difference was observed between tgG-DSE2lim and tgG-DSE2lim PNGase F at any time point, both peaking at week 10–14 and sustaining till endpoint. tgG-DSE5b induced antibody response peaked at week 7 and sustained up to week 18, followed by a trend of decline thereafter.

To characterize the immune response bias of tgG-DSE2lim ± PNGase F and tgG-DSE5b, we quantified the IgG2c and IgG1 antibody titres on week 7 serum samples as a measurement of

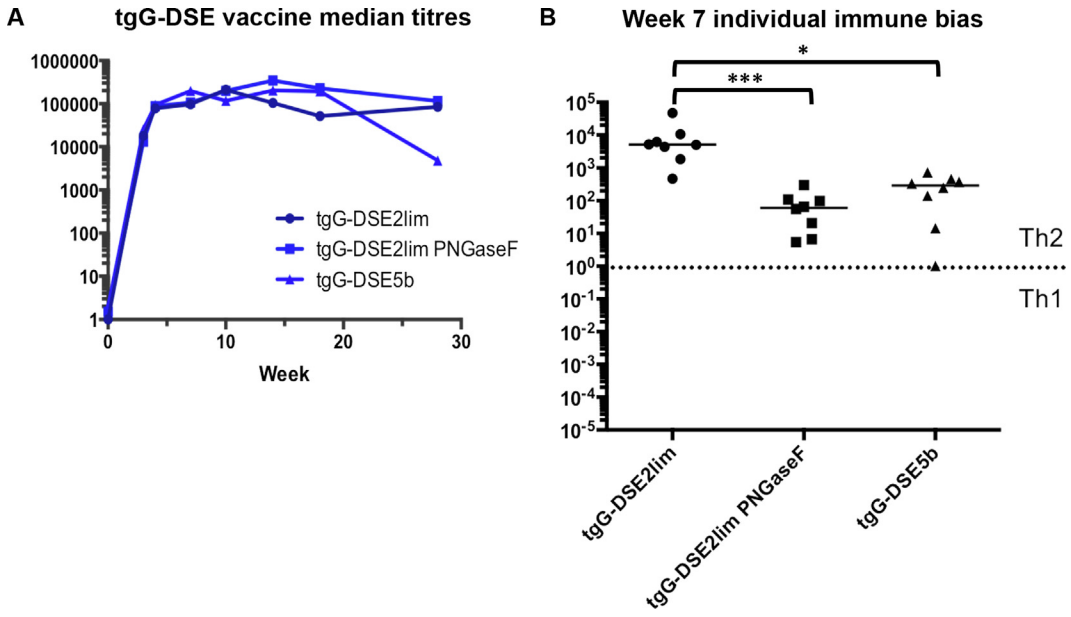

Fig. 1. Epitope-specific antibody titres and immune bias of tgG-DSE2lim ± PNGase F and tgG-DSE5b in vaccinated normal mice by ELISA. (A) Median titres of serum samples from cohorts of vaccinated normal C57BL/6 mice at 3, 4, 7, 10, 14, 18 and 28 weeks following the initial injection (Day 0). (B) Th2/Th1 ratio of IgG1/IgG2c antibody titres of week 7 serum samples from vaccinated C57BL/6 mice. Each dot represents an individual animal. The Kruskal-Wallis and Dunn's multiple comparison tests were performed to identify significant differences. *p*-values of $p < 0.05$ and $p \leq 0.001$ are indicated as * and ***, respectively.

Th1 and Th2 responses, respectively [23] (Fig. S2). Th1 response releases pro-inflammatory cytokines that can be detrimental to the cell, whereas Th2 response promotes a protective antibody production. The ratio of IgG1/IgG2c titres was used as an indirect measure of Th2/Th1 bias [23]. As shown in Fig. 1B, all three vaccines induced desirable Th2-biased responses (Th2/Th1 > 1). In addition, tgG-DSE2lim generated a significantly greater ratio of Th2/Th1 compared to tgG-DSE2lim PNGase F ($p \leq 0.001$).

Together, the above data established that: (1) for all three vaccines, two immunizations were sufficient to elicit robust and sustained desirable Th2-biased antibody responses; (2) the presence of N-glycans in DSE2lim peptide had no effect on the magnitude of tgG-DSE2lim induced antibody responses; (3) tgG-DSE2lim induced a significantly stronger Th2 bias than tgG-DSE2lim PNGase F, and therefore only tgG-DSE2lim was used for subsequent efficacy studies.

2.3. Immunogenicity of tgG-DSE2lim and tgG-DSE5b in hSOD1^{G37R} animals

To characterize the therapeutic effectiveness of tgG-DSE2lim and tgG-DSE5b, we chose an indolent mouse model of ALS, hSOD1^{G37R} line 29, which harbours the human *G37R-SOD1* mutation and resembles symptomatic and pathological clinical features of ALS [24]. Sex-balanced cohorts of 24 pre-symptomatic hSOD1^{G37R} mice and 6 non-transgenic (non-Tg) littermate controls were vaccinated with tgG-DSE2lim, tgG-DSE5b, or control carrier tgG alone beginning at 13 weeks of age (~4 weeks prior to the predicted onset of motor deficit), followed by a boost 3 weeks later. Serum samples were collected 1 week prior to immunization and at weeks 2, 9 and 18 following the initial immunization. As shown in Fig. 2, both vaccines elicited rapid and robust epitope-specific antibody responses as early as week 2. Antibody responses to both vaccines were well sustained up to the latest time point assessed at week 18.

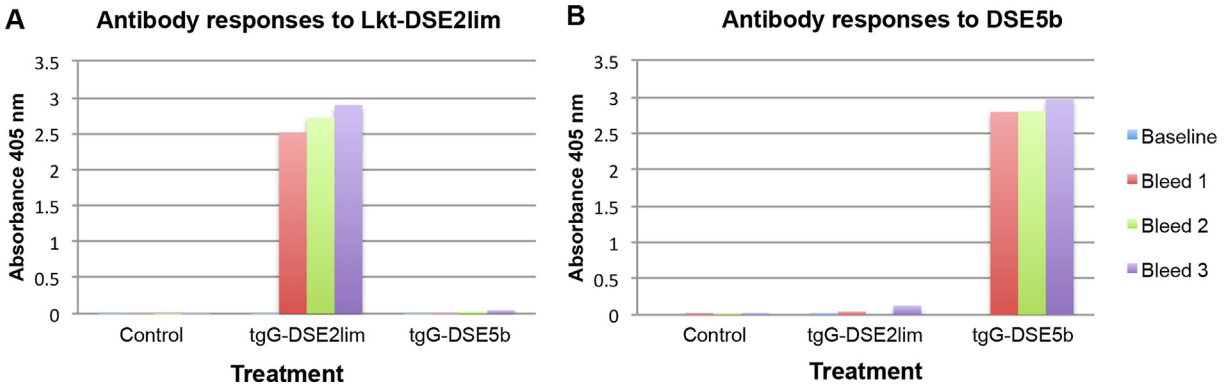

Fig. 2. Epitope-specific antibody responses to tgG-DSE2lim and tgG-DSE5b in vaccinated hSOD1^{G37R} transgenic mice by ELISA. 1:100 diluted serum samples from control tgG, tgG-DSE2lim or tgG-DSE5b vaccinated cohorts collected 1 week prior to (baseline), and 2 (bleed 1), 9 (bleed 2), and 18 (bleed 3) weeks following the initial immunization were analyzed for their epitope-specific antibody responses against coating antigens, Lkt-DSE2lim (A) and DSE5b peptide (B), respectively.

2.4. tgG-DSE5b significantly delayed disease onset and attenuated disease progression in hSOD1^{G37R} animals

To assess the impact of tgG-DSE2lim and tgG-DSE5b on disease trajectory, two well-established behavioural tests, hindlimb extension reflex (HLR) score [25] and rotarod performance [26], were performed on vaccinated hSOD1^{G37R} animals.

HLR scores drop from 4 (healthy) to 0 with disease progression. HLR2 and HLR1 were used as a measurement of disease onset and progression, respectively. As shown in Fig. 3A & B, tgG-DSE2lim had no detectable effects on either disease onset or progression of hSOD1^{G37R} animals. In contrast, tgG-DSE5b not only considerably delayed the disease onset (though it did not reach statistical significance, HLR2 $p = 0.071$) but also significantly slowed disease progression of hSOD1^{G37R} animals compared to control group (HLR1 $p = 0.013$). Control hSOD1^{G37R} animals were ~345 days of age when 30% of the cohort reached HLR2, which was delayed by 70 days in tgG-DSE5b vaccinated group. The effect of tgG-DSE5b on disease progression was especially prominent during end stage. When 75% of hSOD1^{G37R} animals in each cohort reached HLR1, control animals were 524 days of age, which was delayed by an impressive 162 days for tgG-DSE5b vaccinated animals.

The motor function (strength, balance, coordination and endurance) of hSOD1^{G37R} animals was measured using a rotarod apparatus. As shown in Fig. 3C, 30% of control animals reached a rotarod performance failure by 506 days of age, which was delayed by 36 days in tgG-DSE5b vaccinated animals. The rotarod performance of control group continued to deteriorate rapidly thereafter, especially for the following 35 days till 541 days of age, which was significantly ameliorated in tgG-DSE5b treated animals (p = 0.01). In contrast, tgG-DSE2lim had no detectable effect on the motor function of hSOD1^{G37R} animals throughout disease course.

The median age (days) of each cohort in the above clinical analysis is summarized below:

	Control tgG alone	tgG-DSE2lim	tgG-DSE5b
HLR2	422	415	443
HLR1	527	542	548
Rotarod performance	520	541	569

2.5. tgG-DSE2lim and tgG-DSE5b extended the life expectancy of hSOD1^{G37R} mice

Trial endpoint was defined as when the animal was unable to right itself on either side within 30 s after being placed on its side. As shown in Fig. 3D, both vaccines significantly improved the overall survival of hSOD1^{G37R} animals (tgG-DSE2lim $p = 0.045$; tgG-DSE5b $p = 0.037$). The median lifespan of hSOD1^{G37R} was 548 days for control group, which was extended to 598 and

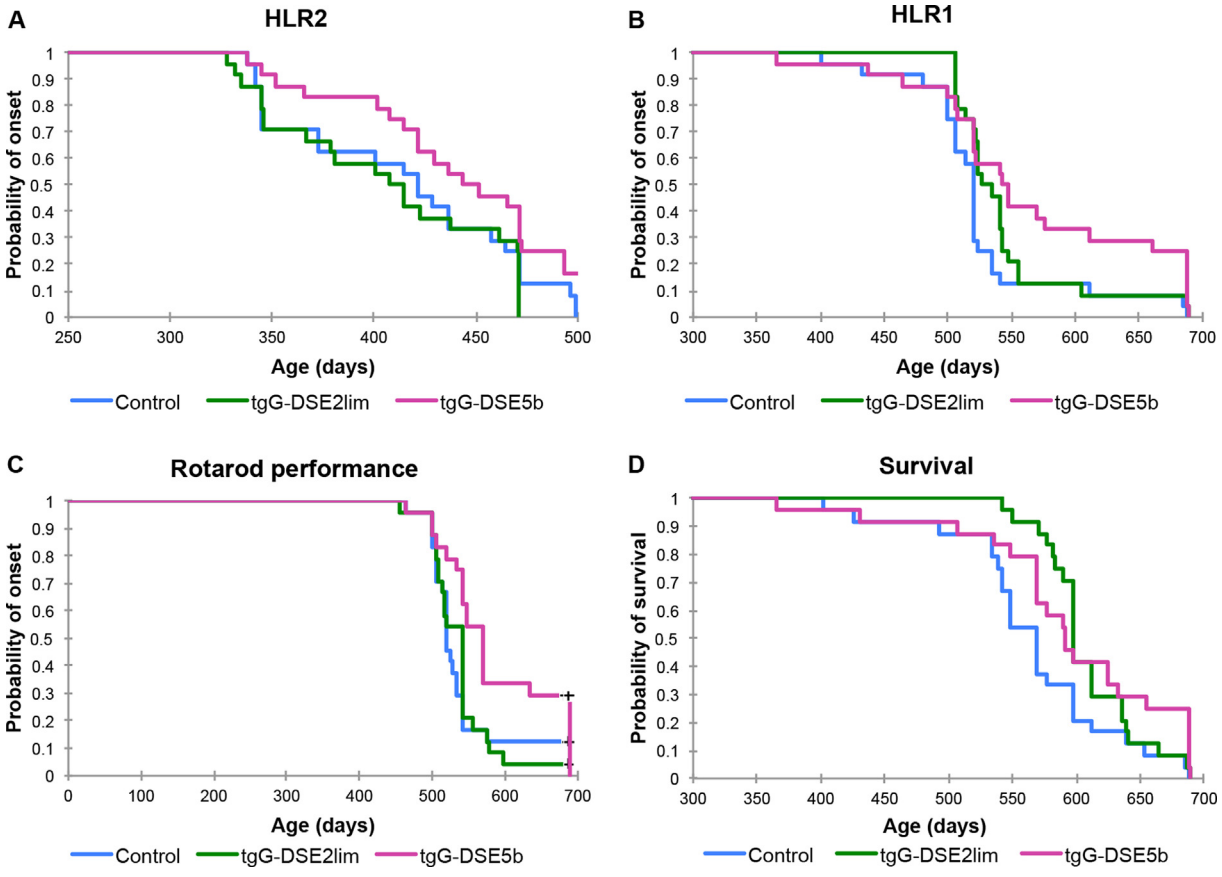

Fig. 3. Therapeutic efficacy of tgG-DSE2lim and tgG-DSE5b on the disease trajectory and lifespan of hSOD1^{G37R} animals. (A) HLR2 shows that tgG-DSE5b, but not tgG-DSE2lim considerably delayed disease onset when compared to control tgG alone, though not reaching statistical significance. tgG-DSE2lim, $p = 0.533$; tgG-DSE5b, $p = 0.071$. (B) HLR1 shows that tgG-DSE5b, but not tgG-DSE2lim significantly slowed progression. tgG-DSE2lim, $p = 0.08$; tgG-DSE5b, $p = 0.013$. (C) Rotarod performance test shows that tgG-DSE5b, but not tgG-DSE2lim significantly improved motor function of hSOD1^{G37R} animals. tgG-DSE2lim, $p = 0.80$; tgG-DSE5b, $p = 0.01$. (D) tgG-DSE2lim and tgG-DSE5b both significantly extended lifespan of hSOD1^{G37R} animals, $p = 0.045$ and 0.037, respectively. Statistics: Kaplan-Meier log-rank tests.

591 days by tgG-DES2lim and tgG-DSE5b vaccination, respectively. Interestingly, although both vaccines significantly improved the life expectancy of hSOD1^{G37R} animals, tgG-DSE2lim exhibited a far greater protection over tgG-DSE5b during earlier symptomatic stage of disease. For example, by day 576, the survival rate of tgG-DSE2lim treated hSOD1^{G37R} was 83.3%, in contrast to 37.5% and 58.3% for control and tgG-DSE5b treated hSOD1^{G37R} animals, respectively. The beneficial effect of tgG-DSE2lim on survival and its lack of efficacy on disease onset is consistent with our findings in a previous passive immunization study using a mouse monoclonal antibody raised against a longer form of DSE2lim [27]. This suggests that DSE2 may be a key region in SOD1 linked to survival and validates the effectiveness of targeting this region on life expectancy in different immunization approaches.

3. Materials and methods

3.1. Animals

hSOD1^{G37R} transgenic mice (line 29) on a C57BL/6-congenic background, B6.Cg-Tg(SOD1*G37R)29Dpr/J, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mouse line was maintained as hemizygotes by breeding with non-transgenic C57BL/6 mice. hSOD1^{G37R} progenies were screened by polymerase chain reaction using primers specific for hSOD1. All procedures were conducted in accordance with the Canadian Council on Animal Care.

3.2. Construction and purification of tgG-DSE immunogens

The sequences of DSE2lim and DSE5b were each presented in a (forward-back-back)$_4$ repeat motif, which we previously found was more immunogenic than the single presentation of the epitope [16]. The gene corresponding to each DSE repeat motif was synthesized by Genscript (Piscataway, NJ, USA) into the pUC57 vector, and then sub-cloned into the tgG eukaryotic expression vector, pEB4.3-tgG-His, to generate tgG-DSE-His.

To express tgG-DSE-His recombinant proteins, HEK293T cells were grown in Dulbecco's Modified Eagle's medium (DMEM) containing 50 μg/ml Gentamycin and 10% FBS. 3×10^5 cells per well of 6-well plates were transfected with 2 μg tgG-DSE-His plasmid using TurboFect transfection reagent (ThermoFisher Scientific, Waltham, MA, USA). Once near confluency, cells were transferred to DMEM selection media containing 2 μg/ml Puromycin, 25 μg/ml Gentamycin. Cultures were gradually scaled up, and 1.5×10^7 cells were then added to 30 ml serum free selection media. Once reaching a density of 2–3 $\times 10^6$ cells/ml, cells were collected and resuspended in 100 ml fresh serum free selection media and incubated with constant shaking. Cultures were maintained at a density of $\sim 4 \times 10^6$ cells/ml. Media was collected daily, clarified, and frozen for a period of 15 days. For protein purification, culture media was concentrated using a centrifugal filter, and tgG-His proteins were purified with Ni-NTA agarose (QIAGEN, Hilden, Germany) following standard procedures. Peak elutions were identified using Bradford aassay, then pooled and dialyzed into phosphate buffered saline (PBS).

3.3. Animal immunization

For each immunogen, 10 μg of the recombinant protein was formulated in PBS with 30% Emulsigen-D for a final injection volume of 100 μl. Each animal received a total of two subcutaneous injections with a 3-week interval.

3.4. Epitope-specific antibody response measurement

Epitope-specific antibody responses were quantified by ELISA as described [16,28], using Lkt-DSE2lim and DSE5b peptide as the coating antigens. For immunogenicity characterization in normal mice, serially diluted serum samples were analyzed. For efficacy studies in hSOD1^{G37R} mice, 1:100 diluted sera were used. Antibody responses were read at 405 nm with a 490 nm reference. Titres were calculated as the reciprocal of the highest dilution with a positive reading 2 standard deviations above the negative control.

3.5. Antibody isotyping

96-well plates were coated with 500 ng per well of appropriate antigen overnight at 4 °C. Plates were washed with PBS containing 0.05% Tween-20 (PBST) and blocked with 0.5% gelatin in PBST for 1 h, followed by incubation with serially diluted serum samples for 2 hrs at room temperature (RT). Plates were then washed and incubated with biotinylated goat anti-mouse IgG2c or IgG1 antibody (1:10,000) in blocking buffer for 1 h at RT. Plates were then washed, incubated with 1:20,000 diluted streptavidin-AP for 45 min at RT, developed with 1 mg/ml PNPP buffer for 2 h, and finally read at 405 nm with a 490 nm reference. Titres were calculated as the reciprocal of the highest dilution with a positive reading 2 standard deviations above the negative control.

3.6. Clinical assessment

Behavioural studies were all conducted blind. HLR score and rotarod performance were performed twice prior to week 0 to serve as learning and conditioning, and then once weekly for the rest of the trial until end point. HLR was scored from 4 to 0 by lifting the animals by the base of the tail. 4: full extension of both hindlimbs for 2 s; 3: full extension of one hindlimb, second limb kicks back and forth; 2: both hindlimbs kick back and forth; 1: one limb kicks back and forth, second limb fully clasped; 0: both limbs fully clasped. Rotarod performance was tested using a rotarod apparatus (IITC Life Science, Woodland Hills, CA, USA). The rotarod was set to begin at 5 rpm, ramping up to 15 rpm over 30 s, then remaining at 15 rpm for 150 s for a total 180 s. If mice failed the test from the first attempt, two more times were repeated. To declare that a mouse was unable to perform the test, they had to fail two weeks in a row. Study endpoint was defined as when the animal was unable to right itself on either side within 30 s after being placed on its side.

3.7. Statistical analysis

One-way ANOVA on a ranked sum was performed to compare epitope-specific antibody titres of tgG-DSE2lim ± PNGase F and tgG-DSE5b in vaccinated normal C57BL/6 mice at each serum collection time point. The Kruskal-Wallis and Dunn's multiple comparison tests were performed to identify significant differences in the Th2 /Th1 bias of tgG-DSE2lim ± PNGase F and tgG-DSE5b induced antibody responses in vaccinated normal C57BL/6 mice. Kaplan–Meier log-rank tests were performed to identify significant differences in the disease onset, progression, and lifespan of tgG-DSE2lim and tgG-DSE5b vaccinated hSOD1^{G37R} animals. *p*-values of $p < 0.05$ is considered significant.

4. Discussion

In the present study, we developed two conformation-based DSE-SOD1 vaccines, tgG-DSE2lim and tgG-DSE5b. Both vaccines elicit robust and sustained antibody responses, and demonstrate

significant therapeutic efficacy on disease trajectory and survival in vaccinated hSOD1^{G37R} mice. Interestingly, tgG-DSE2lim and tgG-DSE5b ameliorate disease phenotypes in a different fashion: tgG-DSE5b substantially delays disease onset and significantly slows disease progression, whereas tgG-DSE2lim essentially shows no effect on disease course. Both vaccines significantly improve the overall life expectancy of hSOD1^{G37R} mice, with tgG-DSE2lim vaccinated animals displaying a ~ 25% greater survival rate than tgG-DSE5b treated during earlier symptomatic stage of the disease. These results indicate that tgG-DSE2lim and tgG-DSE5b influence disease course through different mechanisms.

4.1. DSE2lim and DSE5b may target distinct species of misfolded SOD1 and/or act through different mechanisms

Given the current knowledge that human and mouse SOD1 exhibit little to no interaction *in vivo* [29–31], hSOD1^{G37R} is likely the only source of misfolded SOD1 in hSOD1^{G37R} animals. The kinetics of amyloidogenesis is typically presented by a sigmoid curve consisting of a lag, growth, and eventual plateau phase [13,32,33]. The lag phase is the rate-limiting step for the formation of "seeds", when misfolded polypeptide chains interact, often through hydrophobic regions that are normally masked in natively folded state and form oligomeric β-sheet structures. This is followed by an exponential growth phase, when additional chains continue to be recruited to the "seeds", forming "protofibrils". In the final plateau phase, misfolded chain substrates of the process are depleted, with no further change in β-sheets content. One explanation for the differential effects of tgG-DSE2lim and tgG-DSE5b on disease course and survival is that one DSE may be more accessible than the other during a particular phase of hSOD1^{G37R} amyloidogenesis. That is, DSE5b may be preferably exposed in misfolded "seeds", the predominant species of hSOD1^{G37R} during the lag phase, whereas DSE2lim may be favourably accessible in oligomers and/or fibrils, the predominant species during later phases of amyloidogenesis.

Alternatively, DSE2lim and DSE5b may implicate the existence of at least two conformational species of hSOD1^{G37R} that possess distinct kinetics of amyloidogenesis and consequent differential impacts on disease trajectory. Interestingly, Bidhendi et al. (2016) identified two amyloidogenic strains of mutant SOD1 aggregates isolated from end-stage hSOD1^{G85R} and hSOD1^{D90A} mouse spinal cords [34]. Both strains possessed distinct profiles of structural compositions, *i.e.*, full-length or truncated monomers and larger aggregates. When inoculated into the spinal cords of hSOD1^{G85R} mice, both strains altered disease trajectory differently, reminiscent of the differential clinical outcomes by tgG-DSE2lim and tgG-DSE5b observed in our study.

Future work will be required to dissect the molecular mechanisms by which tgG-DSE2lim and tgG-DSE5b modify disease phenotypes. Understanding early cellular changes in response to tgG-DSE2lim and tgG-DSE5b treatments will potentially lead to the identification of upstream targets that contribute to the pathogenesis of ALS.

4.2. The choice of carrier

To develop an effective peptide-based vaccine against a self-protein, an important factor that can impact the magnitude and duration of antibody responses is the choice of a carrier protein [35,36]. Based on our experience with a peptide-based vaccine development against prion proteins, we here chose tgG over the more commonly used Leukotoxin or gG as the DSE carrier [22]. Our data demonstrates that mere two injections and 10 μg of vaccine each time were sufficient to induce robust antibody responses that were well sustained up to the endpoints evaluated in both pilot studies. The doses and number of injections used in our study were significantly lower than those in previous peptide-based vaccination studies in mouse models of AD, PD, or ALS, where 30–100 μg of immunogens and biweekly-monthly injections throughout disease course were typically applied [10,37–40]. If safe and efficacious, higher antibody production efficiency would obviously be an attractive feature for development of human immunotherapeutics. With readily human-translatable composition and formulation, our design of tgG-DSE based vaccines can expand the therapeutic potential of this platform in eventual human trials.

4.3. Comparison of efficacy from the current study with previous vaccination trials using the hSOD1^{G37R} mouse model

A key element in the development of a safe and efficacious immunotherapeutic for proteinopathies is the design of an epitope(s) that would specifically target the pathogenic species of a disease associated protein while sparing its native conformer. In the earlier ALS vaccination animal trials, recombinant full-length G93A-SOD1 (rec G93A) and misfolding-mimicking metal-free wild-type SOD1 had been used as immunogens [9,11]. While each showed beneficial effects, drawbacks were that the immunogens were large and moreover contain epitopes representative of both native and misfolded SOD1 species, hence limiting the specificity and therapeutic efficacy, and also increasing the probability for cytotoxic pro-inflammatory cytokine release. Therefore, it is crucial to identify the precise DSEs that distinguish pathogenic from native forms of a self-protein in order to maximize the efficacy of targeting disease-associated conforms while minimizing the potential for deleterious autoimmune complications. A major breakthrough came from a study by Liu et al. 2012, where a conformation-based peptide vaccine targeting the epitope "SOD1 Exposed Dimer Interface (SEDI)", was developed and shown to attenuate disease phenotypes in mutant hSOD1 transgenic mice [10]. SEDI is normally buried within the homodimer interface of native SOD1, but exposed upon disruption of the dimeric structure due to mutations or oxidation, and therefore presumably specifically targets unstable monomeric/misfolded SOD1. In the current study, our design strategy of conformational epitopes is expected to not only neutralize existing misfolded SOD1 but also arrest further misfolding propagation by absorbing the "seeds". Comparison of the therapeutic efficacy in the hSOD1^{G37R} model from the current study with previous vaccination trials demonstrates a potential of greater therapeutic efficacy of DSE-based peptide immunogens (tgG-DSE2lim, tgG-DSE5b, and SEDI) over a full-length mutant SOD1 (rec G93A) as an immunogen, *e.g.*, delayed disease onset (Table 1). One plausible explanation is that "rec G93A" vaccination induced polyclonal antibodies that targeted not only misfolded SOD1 but also the native conformer. As such, only a proportion of rec G93A-induced antibodies neutralized misfolded hSOD1 in hSOD1^{G37R} mice, thereby diluting the therapeutic efficacy. This further establishes the advantages of conformation-based DSE vaccines, which would

Table 1
Therapeutic efficacy comparison of tgG-DSE2lim and tgG-DSE5b with immunogens investigated in previous preclinical vaccination studies using the hSOD1^{G37R} model.

	Δ Disease onset[a] (days)	Δ Median life span (days)
Current study	DSE2lim: no effect DSE5b: 36 (506 –> 542. 7.1%)[b]	DSE2lim: 50 (548 –> 598, 9%) DSE5b: 43 (548 –> 591, 8%)
SEDI [10]	28 (318 –> 346, 8.8%)	40 (352 –> 392, 12%)
Rec G93A [9,11]	20 (352 –> 372, 5.4%)	30 (393 –> 423, 7%)

[a] Determined by a 30% drop of motor performance in the Rotorod test.
[b] Control group → vaccinated group, percentage of difference.

not only specifically neutralize misfolded self-proteins without interfering with the normal function of the native conformer, but also minimize "efficacy dilution" and therefore enhance therapeutic effectiveness.

4.4. Immunotherapies for other neurodegenerative diseases

A number of other neurodegenerative diseases share a similar propagating protein misfolding mechanism to ALS, and are characterized with misfolding/aggregation of pathogenic proteins, including amyloid-β (Aβ) and tau in AD, α-synuclein in PD and Lewy body dementia, polyQ repeat expansions in HD, and the prion protein in prion diseases [15]. Misfolded pathogenic proteins in these neurodegenerative diseases not only self-propagate intracellularly, but also can transmit between cells or even tissues [15]. As such, successful immunotherapies specifically absorbing these pathogenic "seeds" would play a key role in both disease initiation and progression. A number of immunotherapy clinical trials have been conducted in an effort to develop treatment for neurodegenerative diseases, which have provided not only critical proof-of-principle data for the feasibility of immunotherapy but also valuable lessons for improvement in the future [14,41–46]. For instance, multiple Phase-III AD trials of Aβ immunotherapies have been conducted within the past two decades, but their immune-targets did not necessarily distinguish the toxic oligomeric form of Aβ (AβOs) from the non-harmful Aβ species (monomers and fibrils) [45,47–49]. These non-specific Aβ immunotherapies trials either failed to demonstrate meaningful efficacy, *e.g.*, bapineuzumab and solanezumab [45]; raised safety concerns such as meningeoencephalitis as reported for the first AD vaccine trial AN1972 [50,51]; or had dose-limiting adverse effects, *e.g.*, aducanumab [52]. This highlights the validity and significance of conformation specificity in the safety and disease-modifying efficacy of the immunotherapeutics against proteinopathies. Moreover, the immune response bias elicited by a vaccine can also have a significant impact on the safety outcome, *e.g.*, the AN1792 trial reported meningoencephalitis in 6% of the patients as a consequence of a Th1 immune response [50]. Therefore, the strong Th2-biased immune responses, in combination with the conformational selectivity of our DSE-based vaccines provide important proof-of-principle data to development of human vaccines with an improved safety and therapeutic/prophylactic outcome.

4.5. Future direction: development of a multivalent vaccine against SOD1 misfolding

Our previous work in development of prion vaccines demonstrates that a multivalent vaccine based on three distinct DSEs in the misfolded form of prion protein, PrP^{Sc}, produces enhanced PrP^{Sc}-specific antibody titres compared to univalent vaccines derived from each individual DSE [17]. This strongly supports the notion that a multivalent DSE vaccination approach may maximize the potential of arresting propagation of pathogenic protein misfolding, and as a result deliver an enhanced therapeutic efficacy. Given the differential efficacy profiles of tgG-DSE2lim and tgG-DSE5b in $hSOD1^{G37R}$ mice reported here, and the positive clinical outcomes from previous passive immunization studies [27], we foresee that a multivalent vaccination approach simultaneously targeting multiple DSEs within SOD1, *e.g.*, DSE2lim, DSE5b, and DSE1a [27], may endow synergistic therapeutic benefits through targeting multiple species of misfolded SOD1 and/or multiple pathogenic mechanisms. Notably, several wild-type-like and metal-binding-region SOD1 mutants identified in fALS have been shown to have significant perturbation in the electrostatic loop of SOD1 [53]. Therefore, the vaccine development strategy established in the current study can also be extended to these mutants in the future for the identification of additional lead epitopes targeting pathogenic SOD1, and ultimately be included in a multivalent vaccine formulation. Moreover, even though the contribution of wild-type SOD1 in ALS pathogenesis remains controversial, misfolded SOD1 has been observed in some post-mortem sporadic ALS (sALS) spinal cords [54]. Therefore, a multivalent DSE-SOD1 vaccine will not only provide a valuable prophylactic/therapeutic intervention for fALS-SOD1 patients and mutant SOD1 carriers, but also benefit at least a proportion of sALS patients. Finally, our findings imply that the conformation-based disease specific vaccination strategy can also be extended to other types of ALS, such as those associated with TDP-43 proteinopathy that is present in the majority of sALS (~90% of all ALS).

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

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